

Original Research

Received 07 June 2026
Revised 18 July 2026
Accepted 01 August 2026

Natural Resources for Human Health 2026, Vol. 6 (14s): 965–973

KEYWORDS:

Cystic fibrosis; Mucin; AlphaFold 3; Papain; Mucolytic; Structure prediction.

Computational and In-Vitro Evaluation of Mucin-Degrading Enzymes for Mucosal Clearance in Cystic Fibrosis

Ruby S.*, Mowriss Benedict, Mugilarasi C, Saravanan. R, N. Mohammed Kaja Muhaideen, A. Mohammed Tanish, K.R. Mohan Ram, A. Murugan

Department of Pharmaceutical Chemistry, Vinayaka Mission's College of Pharmacy, Vinayaka Mission's Research Foundation (Deemed to be University), Salem 636 008, Tamil Nadu, India

*Corresponding author: Ruby S., ruby.s5902@gmail.com

ABSTRACT: Cystic fibrosis airway disease is driven by accumulation of viscous mucus in which the gel-forming mucins MUC5AC and MUC5B determine viscoelasticity. Current mucolytics do not degrade the mucin scaffold, leaving an unmet need for agents acting on the glycoprotein itself. We screened twenty-four candidate mucin-degrading enzymes against four mucin target regions using AlphaFold 3, and evaluated representative enzymes experimentally. Sequences were retrieved programmatically from UniProt and submitted through AlphaFold Server; complexes were ranked by interface predicted TM-score (ipTM). The mucin-selective metalloprotease StcE was included as a positive control. Mucolytic activity was measured against porcine gastric mucin by inclined-slide gravity flow, in triplicate, with N-acetylcysteine as reference standard. None of 101 predicted complexes reached the ipTM threshold of 0.60, and StcE scored 0.32, indicating that the method failed to recognise a known interaction. Predicted confidence segregated by glycan dependence: enzymes cleaving the peptide backbone averaged 0.43 whereas glycan-recognising enzymes averaged 0.22–0.27. Experimentally, all agents reduced flow time dose-dependently. N-acetylcysteine achieved 86.2% reduction, papain 26.1% and α -fucosidase 17.8% (all $p < 0.001$). The protease exceeded the glycosidase significantly ($p < 0.0001$), mirroring the computational ordering. We conclude that protein-only structure prediction cannot model enzyme–mucin association because recognition is glycan-mediated, while confirming that proteolytic degradation reduces mucin viscosity more effectively than glycosidic degradation under these conditions.

Abbreviations

Abbreviation	Definition
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
ipTM	Interface predicted template modelling score
pTM	Predicted template modelling score
PTS	Proline/threonine/serine-rich region

Abbreviation	Definition
NAC	N-acetylcysteine
SD	Standard deviation

1 INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, encoding an epithelial anion channel responsible for chloride and bicarbonate transport (Cho et al., 2023; Patel et al., 2020). Loss of CFTR function dehydrates the airway surface liquid, collapses the periciliary layer and produces thick adherent mucus that obstructs the airways, impairs mucociliary clearance and fosters chronic infection (Wu and Chen, 2024; Graeber and Mall, 2023). This cycle of obstruction, infection and inflammation underlies the progressive lung disease that dominates CF morbidity and mortality (Batson et al., 2022).

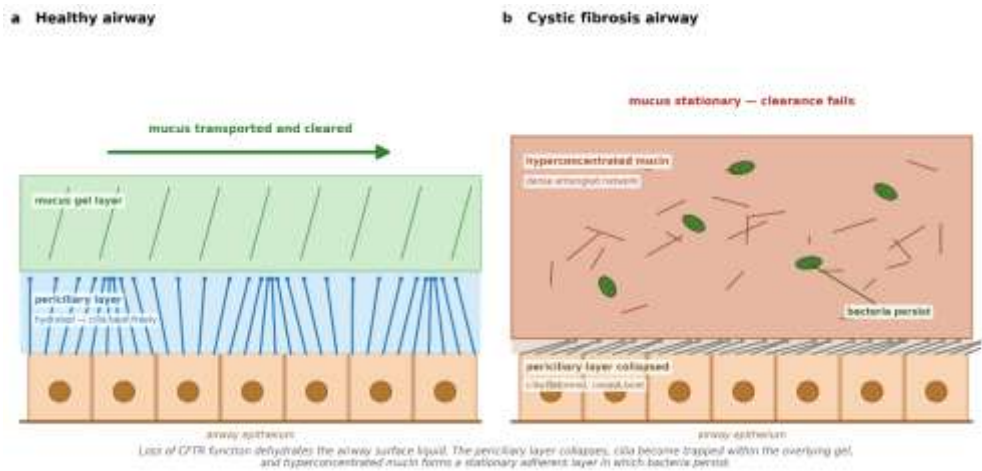


Figure 1 Airway surface architecture in health and in cystic fibrosis. a In the healthy airway, a hydrated periciliary layer allows cilia to beat freely and propel the overlying mucus gel. b Loss of CFTR function dehydrates the airway surface liquid; the periciliary layer collapses, cilia become trapped within the overlying gel, and hyperconcentrated mucin forms a stationary adherent layer in which bacteria persist.

The biophysical properties of airway mucus are governed by the gel-forming mucins MUC5AC and MUC5B, whose concentration, glycosylation and cross-linking determine viscoelasticity (Okuda et al., 2022; Abrami et al., 2024). Both are hyperconcentrated in CF, raising viscosity beyond the capacity of the mucociliary escalator (Abrami et al., 2024; Kato et al., 2022). Structurally, these mucins comprise terminal cysteine-rich multimerisation domains separated by extensive proline/threonine/serine-rich (PTS) regions carrying dense O-linked glycan chains. These glycans may account for the majority of molecular mass and confer the hydration, charge and steric bulk responsible for gel formation (Okuda et al., 2022; Figueira et al., 2022). Reduced sialylation of airway mucin impairs mucus transport, illustrating how directly glycan composition governs function (Harris et al., 2024).

Existing mucoactive agents address this only partially. Dornase alfa degrades extracellular DNA rather than mucin; hypertonic saline rehydrates the airway surface; and N-acetylcysteine (NAC) cleaves disulphide bonds without degrading the mucin backbone or its glycans (Figueira et al., 2022; Menzel and Bernkop-Schnurch, 2018). CFTR modulators correct the channel defect but are ineffective for many genotypes and do not fully reverse established mucus abnormality (Patel et al., 2020; Graeber and Mall, 2023).

Enzymatic degradation offers a mechanistically distinct strategy through two routes. Proteases cleave the mucin peptide core, reducing polymer length and entanglement, whereas glycosidases hydrolyse the O-linked glycans, removing the hydration and charge maintaining the gel (Okuda et al., 2022; Bansil and Turner, 2018). Both occur in nature: breakdown of salivary MUC5B

by dental biofilms requires combined glycosidase and protease action (Wickstrom et al., 2009), gut bacteria express glycosidases that depolymerise mucin O-glycans (Kosciow and Deppenmeier, 2020; Glover et al., 2022), and serine proteases degrade airway mucins in CF (Henke et al., 2011). A distinct class of dedicated bacterial mucinases achieves selectivity by recognising the O-glycan itself; StcE from *Escherichia coli* and IMPa from *Pseudomonas aeruginosa* have both been crystallised bound to O-glycopeptide substrates (Malaker et al., 2019; Bhatt et al., 2021).

Deep-learning structure prediction has altered early-stage screening. AlphaFold2 achieved near-experimental accuracy for single chains (Jumper et al., 2021) and AlphaFold-Multimer extended this to complexes (Evans et al., 2022). AlphaFold 3 introduced a diffusion-based architecture modelling proteins together with nucleic acids, ions and ligands (Abramson et al., 2024). These methods return calibrated confidence estimates; the interface predicted TM-score (ipTM) reports confidence in the predicted interface and provides a principled basis for ranking. A recognised constraint remains: modelling of protein–glycan interaction is context-dependent and predicted glycan conformations do not always preserve correct stereochemistry (Modeling glycans with AlphaFold 3, 2025).

The present study screened twenty-four enzymes spanning five mechanistic classes against the principal CF airway mucins using AlphaFold 3, and evaluated representative enzymes experimentally. A positive control was included by design: StcE, whose mucin degradation is established experimentally (Malaker et al., 2019), permits the distinction between the conclusion that candidate enzymes do not engage mucin and the conclusion that the method cannot detect engagement.

2 Materials and Methods

2.1 Materials

Porcine gastric mucin was supplied by HiMedia Laboratories through Salem Scientific Suppliers, Salem, Tamil Nadu, India. Papain, α -fucosidase and N-acetylcysteine were obtained from the same supplier. All other reagents were of analytical grade.

2.2 Sequence retrieval

Protein sequences were obtained from the UniProt Knowledgebase (The UniProt Consortium, 2025) through its REST interface (<https://rest.uniprot.org>). Retrieval was performed programmatically using custom Python scripts to ensure reproducibility. Accession numbers for the complete panel are given in Supplementary Table S1.

2.3 Enzyme panel

Twenty-four enzymes were selected to represent the principal mechanistic routes of mucin degradation reported in the literature (Wickstrom et al., 2009; Kosciow and Deppenmeier, 2020; Glover et al., 2022; Henke et al., 2011; Malaker et al., 2019; Bhatt et al., 2021), distributed across five classes (Table 1). StcE (UniProt O82882) served as the positive control and IMPa (Q915W4) as a second reference of clinical relevance.

Table 1 Enzyme panel by mechanistic class

Class	Mechanistic role	n
A — Cap-removing glycosidases	Remove terminal fucose, sialic acid and blood-group cap sugars	6
B — Core glycosidases	Hydrolyse inner-chain glycosidic linkages	5
C — Dedicated mucinases	Mucin-selective metalloproteases	6
D — Host airway proteases	Proteases present in the CF airway	5
E — Generic proteases	Broad-specificity mechanistic baselines	2

2.4 Target preparation

MUC5AC (UniProt P98088) and MUC5B (Q9HC84) were selected as the principal gel-forming airway mucins. Because neither has been characterised structurally in its entirety, two complementary regions were defined for each.

The PTS O-glycopeptide region was identified computationally by sliding-window analysis of the full-length sequence, using a 120-residue window advanced in 10-residue steps and scored by combined threonine, serine and proline content. The highest-scoring window was selected, yielding regions of 83% and 68% combined content for MUC5AC and MUC5B respectively. A 400-residue segment of the D1–D2–D'D3 multimerisation region was taken as the N-terminal domain target.

2.5 Complex prediction

Each enzyme sequence was paired with each target sequence and assembled into AlphaFold Server batch submission format by custom script, with the two chains declared as separate protein entities. Files were uploaded to AlphaFold Server (<https://alphafoldserver.com>) and predictions executed using AlphaFold 3 (Abramson et al., 2024) with default server settings. Each completed job returned predicted coordinates in mmCIF format together with confidence data in JSON format.

2.6 Evaluation of predicted complexes

Downloaded archives were processed by custom Python scripts that traverse the directory tree, pair each structure with its confidence file and extract ipTM and pTM values. Complexes were interpreted using established confidence bands: above 0.80, high confidence; 0.60–0.80, likely complex; 0.40–0.60, uncertain; below 0.40, unlikely to represent a genuine interaction. A threshold of ipTM ≥ 0.60 was specified in advance for advancing a candidate.

2.7 In-vitro mucolytic assay

Synthetic mucin substrate was prepared by dissolving porcine gastric mucin in phosphate buffered saline (pH 7.4) to 2.0% w/v. Stock solutions were stored at 4 °C and equilibrated to 37 °C before use.

Because the substrate was too viscous for capillary viscometry, relative viscosity was assessed by inclined-slide gravity flow. A glass slide was fixed at 40°, a fixed volume of sample applied at the upper mark, and the time taken to travel to the lower mark recorded. Longer flow time indicates higher viscosity. Test agents were N-acetylcysteine (0.5–2.0 g/100 mL) as reference standard, papain (0.5–2.0 mg/mL) representing proteolytic degradation, and α -fucosidase (0.5–2.0 U/mL) representing glycosidic degradation. Samples were incubated at 37 °C for 30 min before measurement. Untreated mucin served as control. Every condition was measured in triplicate ($n = 3$).

2.8 Statistical analysis

Results are expressed as mean $\pm$ standard deviation of three independent measurements. Percentage reduction in flow time was calculated relative to the untreated control mean. Comparisons against control, and between papain and α -fucosidase at matched concentration, used Welch's unpaired two-tailed t-test. A value of $p < 0.05$ was considered significant. Analyses were performed in Python using the SciPy library.

3 RESULTS AND DISCUSSION

3.1 Predicted complexes

One hundred and one enzyme–mucin complexes were predicted and scored. No complex reached the pre-specified ipTM threshold of 0.60; the highest value observed was 0.56 for neutrophil elastase against the MUC5B N-terminal domain.

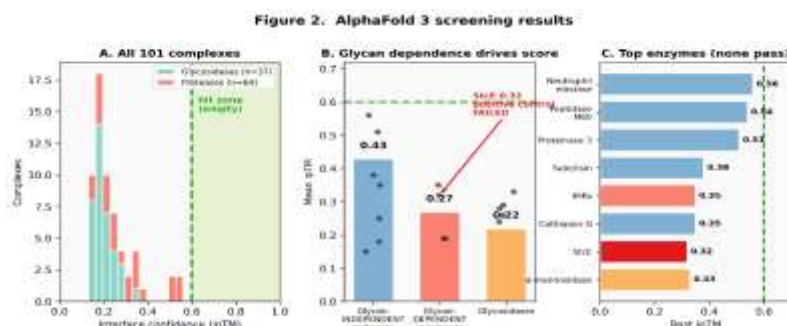


Figure 2 Predicted interface confidence across the enzyme panel. a Distribution of interface predicted TM-score (ipTM) across all 101 predicted complexes; the region above the 0.60 threshold contains no complexes. b Mean ipTM grouped by

glycan dependence, with individual complexes overlaid; the StcE positive control is indicated. c Highest ipTM achieved by each enzyme.

3.2 Positive control

StcE achieved a maximum ipTM of 0.32 across all four target regions, and IMPa a maximum of 0.35. Both fall within the band interpreted as unlikely to represent a genuine interaction, despite both enzymes being established mucin-degrading proteases with published structures in complex with mucin-derived O-glycopeptides (Malaker et al., 2019; Bhatt et al., 2021).

3.3 Relationship between glycan dependence and predicted confidence

Predicted confidence was not distributed randomly. Grouping enzymes by whether engagement with mucin requires O-glycan recognition revealed a systematic difference (Table 2). Enzymes cleaving the peptide backbone without requiring glycan recognition achieved a mean ipTM of 0.43, whereas mucin-selective mucinases and glycosidases achieved 0.27 and 0.22 respectively.

Table 2 Predicted interface confidence by glycan dependence

Enzyme group	Requires glycan recognition	Mean ipTM	Highest ipTM
Generic and host proteases	No — cleave peptide backbone	0.43	0.56
Mucin-selective mucinases	Yes — recognise O-glycans	0.27	0.35
Glycosidases	Yes — glycans are the substrate	0.22	0.33

3.4 In-vitro mucolytic activity

Untreated mucin gave a mean flow time of 242.43 ± 2.14 s. All three agents produced concentration-dependent reductions in flow time

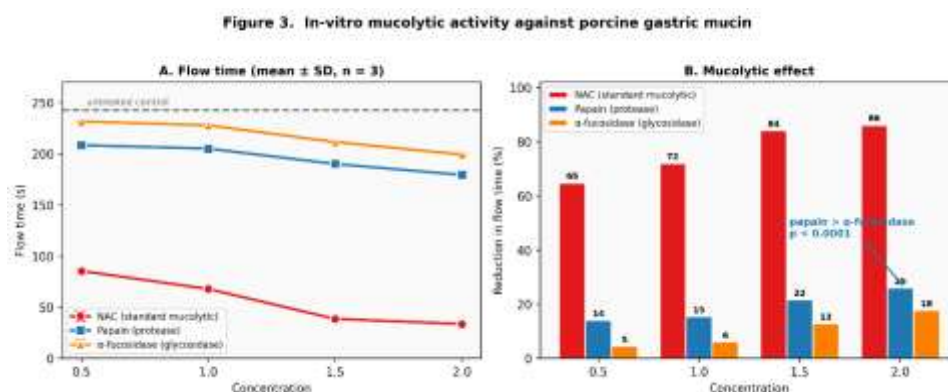


Figure 3 In-vitro mucolytic activity against 2.0% w/v porcine gastric mucin. a Flow time as a function of concentration for N-acetylcysteine, papain and α -fucosidase; the dashed line indicates the untreated control. b Percentage reduction in flow time relative to control. Values are mean $\pm$ SD of three independent measurements.

Table 3 In-vitro mucolytic activity (mean $\pm$ SD, n = 3)

Treatment	Concentration	Flow time (s)	Reduction (%)	p vs control
Untreated control	—	242.43 $\pm$ 2.14	—	—
N-acetylcysteine	0.5 g/100 mL	85.30 $\pm$ 3.02	64.8	7.49×10^{-7}
N-acetylcysteine	1.0 g/100 mL	67.70 $\pm$ 2.20	72.1	6.40×10^{-8}

Treatment	Concentration	Flow time (s)	Reduction (%)	p vs control
N-acetylcysteine	1.5 g/100 mL	38.47 ± 0.57	84.1	1.14×10^{-5}
N-acetylcysteine	2.0 g/100 mL	33.47 ± 1.36	86.2	1.58×10^{-7}
Papain	0.5 mg/mL	208.29 ± 0.81	14.1	3.63×10^{-4}
Papain	1.0 mg/mL	204.93 ± 0.63	15.5	4.67×10^{-4}
Papain	1.5 mg/mL	189.96 ± 0.21	21.6	4.96×10^{-4}
Papain	2.0 mg/mL	179.25 ± 1.19	26.1	1.69×10^{-5}
α -Fucosidase	0.5 U/mL	231.43 ± 0.90	4.5	5.62×10^{-3}
α -Fucosidase	1.0 U/mL	227.70 ± 0.70	6.1	3.69×10^{-3}
α -Fucosidase	1.5 U/mL	211.33 ± 0.61	12.8	7.56×10^{-4}
α -Fucosidase	2.0 U/mL	199.17 ± 1.32	17.8	3.64×10^{-5}

N-acetylcysteine reduced flow time from 242.43 s to 33.47 ± 1.36 s at 2.0 g/100 mL, a reduction of 86.2% ($p = 1.58 \times 10^{-7}$). Papain reduced flow time to 179.25 ± 1.19 s at 2.0 mg/mL, a reduction of 26.1% ($p = 1.69 \times 10^{-5}$). α -Fucosidase reduced flow time to 199.17 ± 1.32 s at 2.0 U/mL, a reduction of 17.8% ($p = 3.64 \times 10^{-5}$). All reductions were statistically significant at every concentration tested.

At matched concentration, papain produced significantly greater reduction than α -fucosidase (26.1% versus 17.8%; $p < 0.0001$).

3.5 Discussion

The computational screen produced no hits. Interpreted alone this would be ambiguous, since it could indicate either that the enzymes do not engage mucin or that the method cannot detect engagement that occurs. The positive control resolves the ambiguity. StcE degrades mucin, established by direct experiment including crystallography of the enzyme bound to mucin glycopeptide (Malaker et al., 2019), yet scored 0.32. A method that fails to recognise an interaction known to occur cannot be relied upon to identify interactions that are unknown. The appropriate interpretation is therefore a limitation of the method rather than an absence of biology.

The distribution of scores indicates the cause. Enzymes were separated by whether they require glycan recognition to engage mucin, and those that do not scored approximately twice as high as those that do. This is the pattern expected if the glycans were absent from the models, which they were: AlphaFold 3 was used in its default protein-only configuration, so the mucin targets were submitted as bare peptide sequences. Native mucin carries dense O-linked glycan chains along the PTS region, and for glycan-recognising enzymes those chains constitute the binding determinant. StcE therefore fails for the same reason as the glycosidases; it is selective for mucin precisely because it reads the glycan. The residual signal retained by neutrophil elastase and proteinase 3 is consistent, since these enzymes cleave peptide bonds without requiring glycan recognition.

A second factor applies to the PTS targets specifically. Structure prediction derives accuracy from evolutionary information contained in multiple sequence alignments, and the PTS region is a low-complexity tandem repeat against which alignment is poor. The mucin chain is consequently modelled with low per-residue confidence, which independently limits confidence in any interface involving it. This compounds but does not replace the absence of glycans, since the N-terminal domain targets, which align deeply, also yielded no hits for glycan-dependent enzymes.

The experimental data are consistent with the computational ordering at the level of mechanistic class. Papain, representing proteolytic degradation, produced significantly greater reduction in mucin viscosity than α -fucosidase, representing glycosidic degradation, at matched concentration. The computational screen likewise ranked backbone-cleaving proteases above glycan-

recognising enzymes. This concordance suggests that although absolute predicted confidence is unreliable for this target class, the relative ordering by degradation route retains some information.

Both enzymes were substantially less effective than N-acetylcysteine, which reduced flow time by 86.2%. This is expected: NAC acts by cleaving disulphide bonds that cross-link mucin polymers, a mechanism producing rapid gel collapse, whereas enzymatic degradation of the backbone or glycans proceeds more gradually under the conditions tested. The comparison establishes assay sensitivity rather than therapeutic equivalence.

Three misinterpretations should be guarded against. These data do not indicate that the enzymes fail to degrade mucin; several are documented to do so, and papain and α -fucosidase both reduced viscosity significantly here. They do not indicate that enzymatic mucolysis is unpromising, since the biological rationale is unaffected by a limitation of structure prediction. Finally, they do not represent a failed screen: a screen incorporating a functioning control that returns an interpretable negative result has fulfilled its purpose.

The wider implication concerns application of structure prediction to glycoproteins. Mucins are among the most heavily glycosylated proteins known, but the reasoning extends to any target where recognition is carbohydrate-mediated. Where the binding determinant is the glycan, protein-only prediction models the wrong molecule. This argues not against structure prediction in such systems but for mandatory inclusion of positive controls, without which a negative result cannot be distinguished from a methodological blind spot. These findings are consistent with systematic evaluation of AlphaFold 3 in glycobiology, which reports highly context-dependent performance on protein–glycan interactions (Modeling glycans with AlphaFold 3, 2025).

Limitations of the present work should be acknowledged. The synthetic substrate is a simplified mucin–buffer system lacking the DNA, lipids and cellular material of native CF mucus. Porcine gastric mucin differs from airway mucin in glycosylation, and inclined-slide gravity flow provides a relative rather than absolute measure of viscosity; rheometry would be preferable. Each complex was represented by a single static model without conformational sampling, and target regions represent defined segments of much larger glycoproteins.

4 CONCLUSION

Twenty-four candidate mucin-degrading enzymes were screened against MUC5AC and MUC5B using AlphaFold 3, yielding 101 predicted complexes, none of which reached the confidence threshold. Failure of the StcE positive control, together with the systematic relationship between glycan dependence and predicted confidence, indicates that protein-only structure prediction cannot model enzyme–mucin association because the principal determinant of recognition is the O-linked glycan, absent from the models.

Experimentally, papain and α -fucosidase both reduced mucin viscosity significantly and concentration-dependently, with the protease exceeding the glycosidase, mirroring the computational ordering by mechanistic class. Computational prioritisation of mucin-degrading enzymes therefore requires glycan-aware structural methods, positive controls should be considered mandatory in structure-prediction screens, and experimental evaluation of enzymatic mucolysis remains warranted.

Statements and Declarations

Competing Interests The authors declare no competing financial or non-financial interests.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Author Contributions :

Ruby S.: Supervision, Conceptualization.

Saravanan R.: Supervision, Methodology guidance.

Mowriss Benidict, Mugilarasi C.: Conceptualization, Investigation, Software, Formal Analysis, Writing – Original Draft (lead technical authors).

N. Mohammed Kaja Muhaideen, A. Mohammed Tanish, K.R. Mohan Ram, A. Murugan: Writing – Review & Editing, Data Curation, Documentation.

Data Availability All data generated or analysed during this study are included in this published article and its supplementary information files. Analysis scripts are available from the corresponding author on reasonable request.

Ethics Approval Not applicable. This study did not involve human participants or animals.

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